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Comprehensive Kinetic and Structural Studies of Different Flavonoids Inhibiting Diphenolase Activity of Mushroom Tyrosinase[1]

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Abstract—The effect of 4 flavonoids on the diphenolase activity of mushroom tyrosinase was studied using spectroscopic approach. Analysis of kinetic data demonstrated that flavonoids cause a reversible inhibition of the enzyme activity. Further study showed that gallic acid acted as noncompetitive inhibitor, whereas chrysin, naringin and quercetin inhibited the diphenolase activity of mushroom tyrosinase in a competitive fashion. Comparison of the inhibition constants revealed that the strength with which the inhibitors acted on the enzyme activity was ranking as follows: chrysin (K_i 7.90 mM) < quercetin (K_i 7.44 mM) < naringin (K_i 3.04 mM) < gallic acid (K_i 1.5 mM). These data, therefore, suggest that gallic acid is the most potent inhibitor of the enzyme compared to the other flavonoids used.

Keywords: inhibition, kinetics, structure, mushroom tyrosinase, flavonoids

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Tyrosinase (EC.1.14.18.1) is the ubiquitous enzyme involved in the biosynthesis of melanine in a copper-dependent manner [1, 2]. This enzyme catalyzes the hydroxylation reactions leading to the formation of melanins through 3,4-dihydroxyphenylalanine (L-DOPA) and L-DOPA-quinone, using L-Tyr as substrate [3]. Melaninis are engaged in the biosynthesis of hair-color products as well as betalains, the sclerotization of insect cuticle, and defense responses in arthropods, plants, and fungi [4, 5].

From clinical point of view, the inhibition of tyrosinase to treat pigmentation disorders has been an important subject to study [6]. The inhibition of melanogenesis using natural products through blockade of adenylyl cyclase, could be a promise in development of skin remedies and cosmetic products for hyperpigmentation [7]. Hence tyrosinase, one of the signaling molecules involved in the adenylyl cyclase signaling pathway, has recently gained much attention as a potential target for finding agents with the aim of skin depigmentation [3]. It has been previously reported that cupferron, flavonoids, hexylresorcinol, dodecylresorcinol and alkylbenzaldehydes inhibit the enzymatic oxidation of DOPA [8]. Previous studies have revealed that the natural flavonoids, including 5,7-dihydroxyflavone (chrysin), extracted from plants, honey, and propolis, inhibit tyrosinase very effectively in a reversible manner [1, 6]. It has been shown that some flavonoids, the derivatives of benzopyrone, act as copper-chelating inhibitors, inactivating tyrosinase [9, 10]. Additionally, some other flavonoids reported to competitively inhibit L-DOPA oxidation by mushroom tyrosinase (MT) [11]. Despite the fact that flavonoids reversibly inhibit MT [11], the mechanisms of inhibition by which they act on the enzyme activity are yet to be understood. In continuation of our previous researches on MT activity and stability [12–15], in the present work, we carried out comprehensive kinetic and structural studies using spectroscopic methods to understand the mechanisms through which some natural falvonoids, including quercetin, chrysin, naringin, and gallic acid (Fig. 1) inhibit the diphenolase activity of MT activity. Our data clearly demonstrated that the applied flavonoids reversibly inhibit diphenolase activity of MT. Importantly, despite the close structural similarity between the flavonoid derivatives used, these compounds showed differences in their mechanism of inhibition.

MATERIALS AND METHODS

Chemicals. MT, DOPA, Na_2HPO_4, NaH_2PO_4, chrysin, gallic acid, quercetin, and naringin were obtained from Sigma (USA).

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Kinetic analysis of tyrosinase inhibition. Mushroom tyrosinase (MT) activity was measured by following the oxidation reaction of L-DOPA to L-DOPA-quinone at 475 nm (dopachrome accumulation wavelength) and 20°C using the molar absorption coefficient of 3700 $M^{-1}cm^{-1}$ [8]. Absorption measurements were accomplished utilizing a UV-2100 spectrophotometer (Cary eclipse model bio 100 Varian Co., Australia). The diphenolase reaction of MT was performed in 50 mM phosphate buffer (pH 6.8) at 20°C [1, 8]. An aliquot of 30 μL aqueous solution of MT (100 units), L-DOPA (0.3, 0.6, 0.9 or 1.2 mM) as a substrate, and each inhibitor taken in various concentrations were added to reaction mixture (the final volume of 1 mL). The rate of dopachrome development was determined at OD_{475}. The kinetic factors including K_M and V_{max} were obtained for MT by the double-reciprocal plots of Lineweaver-Burk.

Intrinsic fluorescence studies. The fluorescence emission spectra were monitored using a Cary 100 Bio model spectrofluorimeter with the excitation wavelength of 280 nm for intrinsic Trp fluorescence measurements, and the emission wavelength ranged between 300 and 450 nm. The quenching rate parameters for MT was obtained upon addition of chrysin, naringin, quercetin or gallic acid taken in various concentrations using Stern-Volmer plots [16]. To examine the interaction of flavonoids with MT, the fluorescence spectra were inspected from 300 to 450 nm following excitement at 280 nm. Fluorescence emission spectra were recorded at 2 temperatures, i.e., 298 and 310 K upon excitation. The slit widths of 5 nm were used for both excitation and emission experiments. To figure out the fluorescence quenching mechanism, the quenching data for examined flavonoids were firstly evaluated using the classical Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Q], \qquad (1)$$

where F and F_0 are the intensity of fluorescence in the presence and absence of quencher, K_{SV}, the Stern-Volmer quenching constant, and [Q], the quencher concentration.

In general, if the plots of this equation display a straight association, a purely collisional quenching process or static quenching process would be inferred [17]. But, if the Stern-Volmer plots show deviation from linear state, the data shall be process according to an adapted Stern-Volmer equation:

$$\frac{F_0}{(F_0 - F)} = \frac{1}{f_a} + \frac{1}{f_a K_{SV}}\frac{1}{[Q]}, \qquad (2)$$

where f_a is the early fluorescence fraction accessible to quencher which is not necessarily related to the proportion of Trp residue available to quenching [18]. $F_0/(F_0-F)$ is in linear relation with the reciprocal quencher concentration (1/[flavonoides]), f_a can be obtained from the values of intercept and K_{SV} from the slope. The binding parameters can be calculated according to the following equation:

$$\log\left[\frac{F_0 - F}{F}\right] = \log K_A + n\log[Q], \qquad (3)$$

where n represents the number of attachment positions, K_A means the attachment constant or the apparent union constant for quencher-enzyme association, and F and F_0 are the intensity of fluorescence in the presence and absence of quencher(Q), respectively.

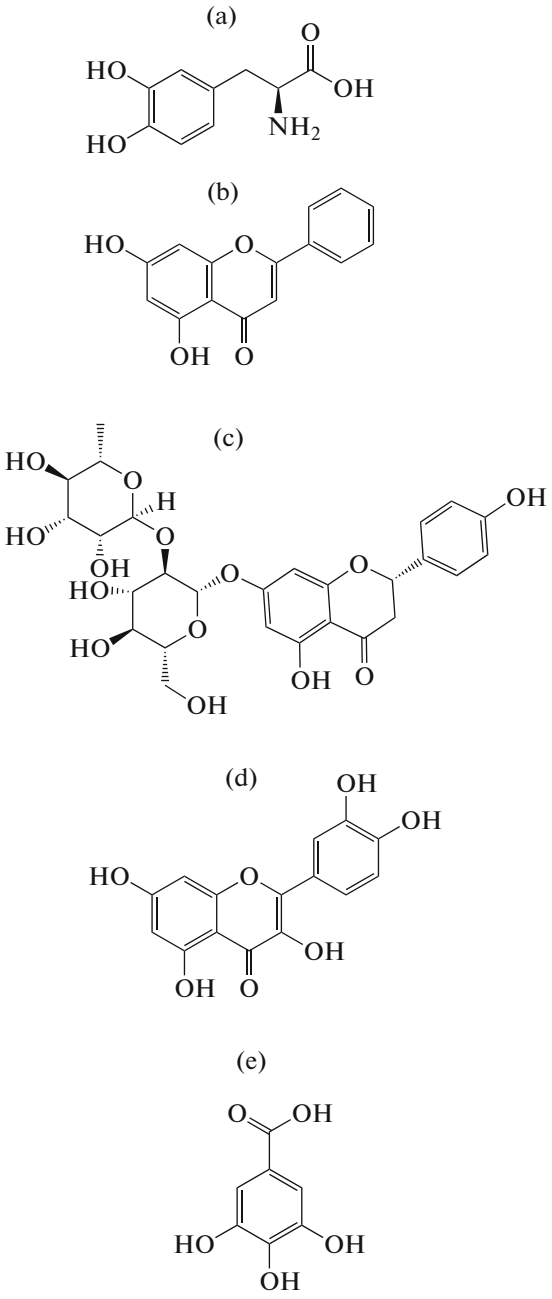

Fig. 1. Structures of L-DOPA (a), chrysin (b), naringin(c), quercetin (d), and gallic acid (e).

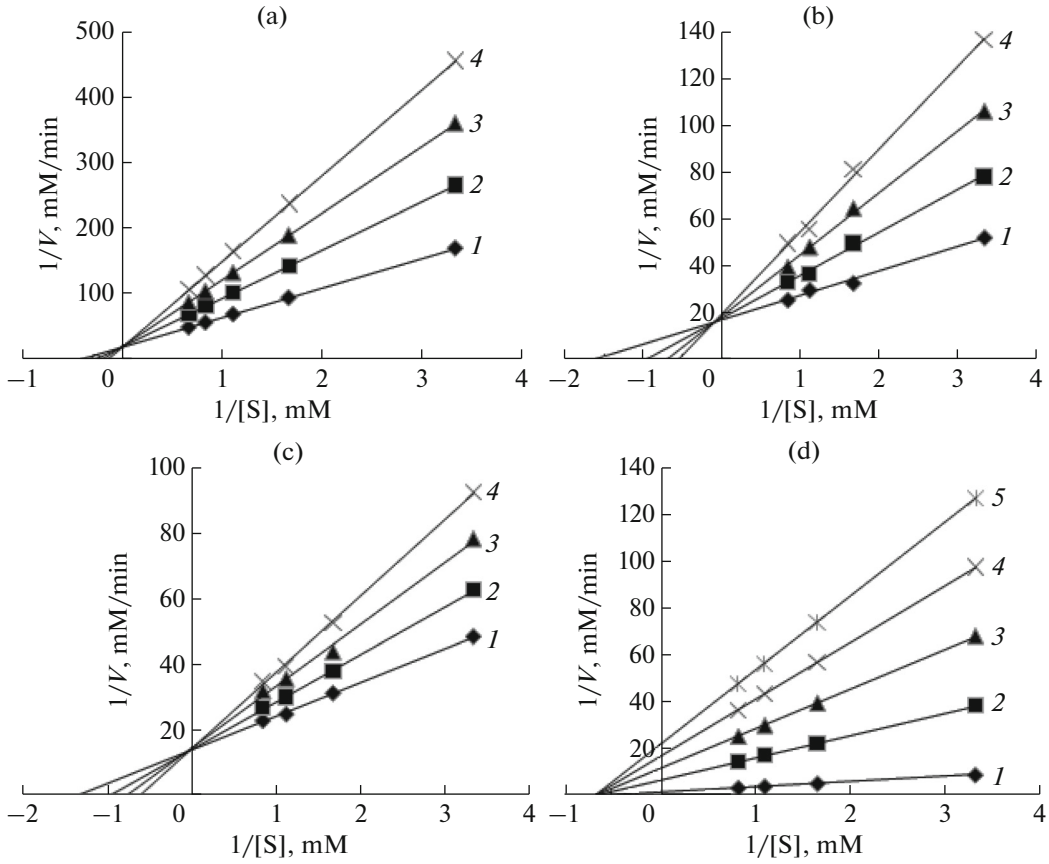

Fig. 2. Lineweaver-Burk plots for diphenolase activity of MT with L-DOPA as substrate and 23.16 μM of the enzyme, in the presence of different concentrations of chrysin (a): 0 (*1*), 5 (*2*), 10 (*3*) and 15 mM (*4*); naringin (b): 0 (*1*), 2.5 (*2*), 5 (*3*) and 7.5 mM (*4*); quercetin (c): 0 (*1*), 5 (*2*), 7.5 (*3*) and 10 mM (*4*); gallic acid (d): 0 (*1*), 5 (*2*), 10 (*3*), 15 (*4*) and 20 mM (*5*).

Circular dichroism measurement. The secondary structure of MT was analyzed in far UV region (195–260 nm) by an Aviv model 215 spectropolarimeter (Lakewood, USA). Far UV CD spectra of MT was studied at the concentration of 0.21 mg/mL with 1 mm path length quartz cell. Enzyme solutions were prepared in the 50 mM PBS buffer (pH 6.8). The ellipticity for the enzyme solutions were obtained in the presence of chrysin, naringin, quercetin, and gallic acid, after 5 min incubation. All spectra were measured in triplicate from 195 to 260 nm and a back ground-correction method was performed against buffer as blank. The data were smoothed by applying the software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without distorting their peak shapes.

RESULTS

Chrysin, naringin and quercetin inhibited the diphenolase activity of MT competitively. The inhibition kinetics of chrysin, naringin, and quercetin on diphenolase activity of MT were examined and the kinetic parameters were obtained from Lineweaver–Burk plots (Fig. 2a–2c). From our kinetic data, it appeared that the flavonoids inhibited the enzyme activity reversibly in a competitive fashion. This is evidenced by the fact that increasing the concentration of inhibitors led to a set of lines which differed only in their slopes (Fig. 2a–2c).

Gallic acid inhibited the diphenolase activity of MT in a noncompetitive fashion. To study the effect of gallic acid on kinetic performance of the diphenolase activity of MT, the oxidation reaction of L-DOPA was monitored following treatment with gallic acid and in untreated control. From Lineweaver-Burk plots (Fig. 2d), the kinetic data revealed that, under the settings used in this study, the oxidation reaction of L-DOPA obeyed the Michaelis–Menten kinetics. These data also demonstrated that gallic acid inhibited the enzyme activity in a noncompetitive manner since increasing concentration of gallic acid led to a set of lines with different slopes, intersecting one another at a common point on the *X*-axis.

Effect of flavonoids on the tyrosinase structure. The effect of flavonoids on tyrosinase structure was investigated by measuring changes in the intrinsic protein fluorescence after flavonoid treatment. Fluorescence data indicate that flavonoids induce a quenching effect which tendency to increase as the concentration

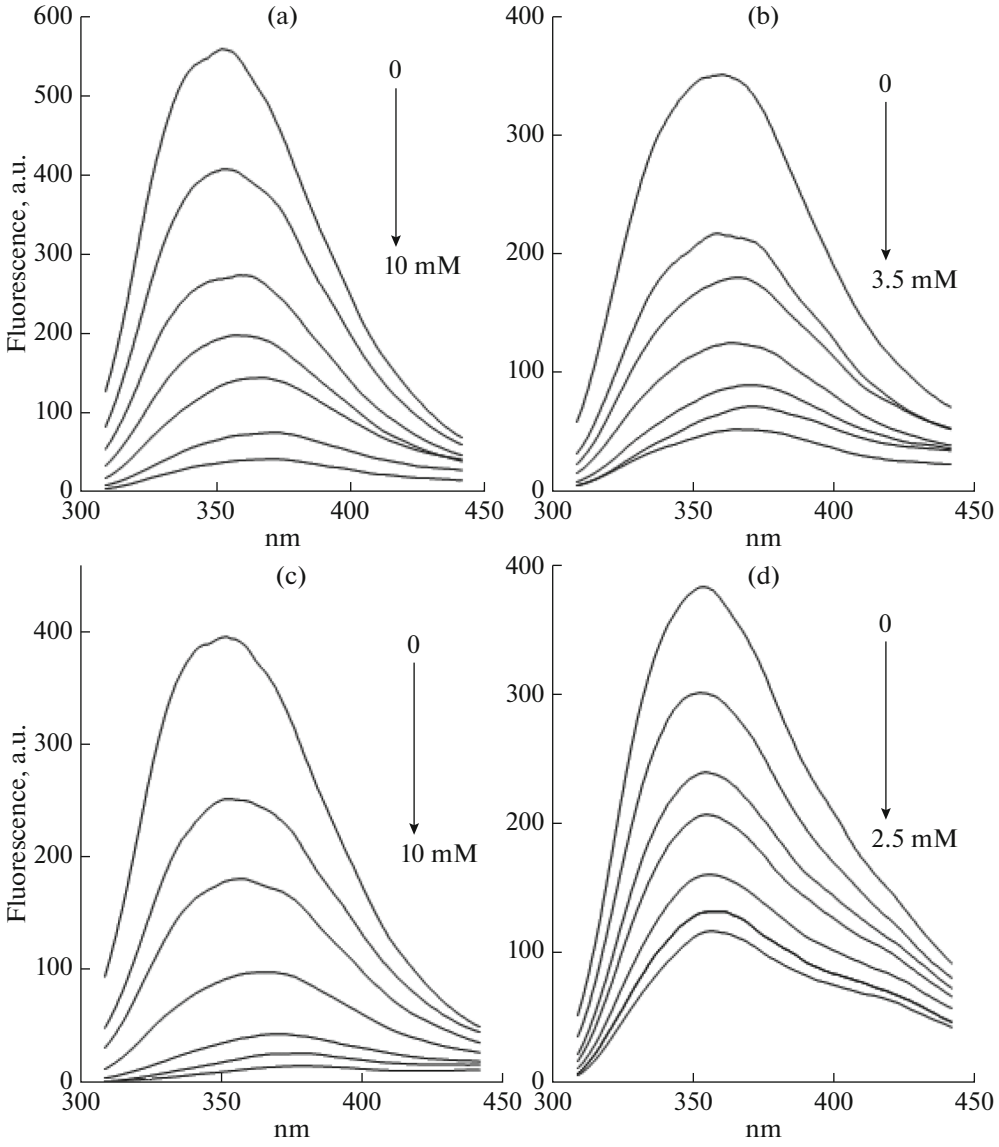

Fig. 3. Fluorescence quenching spectra of MT at various quantities of chrysin (a), naringin (b), quercetin (c) and gallic acid (d).

of inhibitors (flavonoids) increased (Fig. 3). Comparing the spectral maximum peak wavelength (λ_{max}) also revealed that the flavonoid derivatives quenched the intrinsic fluorescence of MT. This decline in the intensity of fluorescence was coupled with the loss of activity in the enzyme. The fluorescence spectra of MT traced with rising amounts of chrysin, naringin, quercetin and gallic acid (Fig. 3). As shown in Fig. 3, the intensity of fluorescence for MT diminished frequently with rising flavonoids concentration whilst emission peaks as well as their profiles stay roughly unaffected. The results suggest that flavonoid interaction with MT might occur without any alteration in the milieu surrounding Trp.

Stern-Volmer study. To establish the means of interaction between MT and flavonoids, fluorescence intensity records were analyzed with the Stern-Volmer equation. Fluorescence quenching includes static and dynamic quenching [18]. Dynamic quenching occurs in association with collision between fluorophore in its excited state and quencher. Static quenching is caused by binding of quencher to fluorophore in its ground state [19] and results in linear Stern-Volmer plots. However, the Stern-Volmer plots for interaction of flavonoids with MT illustrated a slight positive divergence (Fig. 4), signifying the occurrence of static quenching in addition to dynamic quenching. The values of Stern-Volmer quenching constant were estimated from equation 2 and presented in table. The interaction forces between chrysin, naringin, quercetin and gallic acid and MT like the other interactions of proteins and

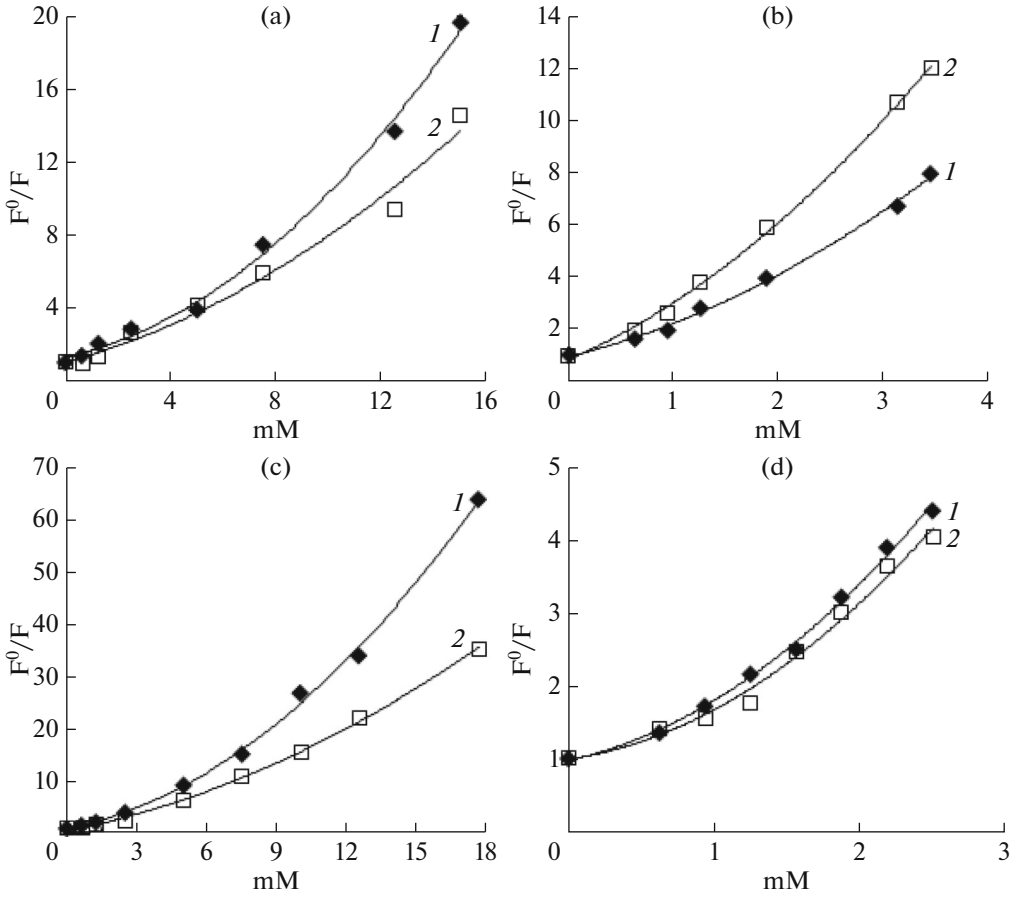

Fig. 4. Stern-Volmer plots for the interaction of chrysin (a), naringin (b), quercetin (c), and gallic acid (d) with MT at 298 (*1*) and 310 K (*2*). The original data have been used from fluorescence data in Fig. 3.

drugs comprise weak forces e.g. hydrophobic, electrostatic, and hydrogen bonds [20]. To identify what force(s) act between flavonoids and MT, the thermodynamic parameters i.e., changes in the enthalpy ($\Delta H°$), free energy ($\Delta G°$), and entropy ($\Delta S°$) were estimated for interactions through equations (4), (5) and the van't Hoff equation [21]:

$$\mathrm{Ln}K = -\Delta H/RT + \Delta S/R, \quad (4)$$

$$\Delta G = \Delta H - T\Delta S = -RT\mathrm{Ln}K, \quad (5)$$

Inhibition constants (K_i), binding and thermodynamic parameters calculated for the interaction of different flavonoids with diphenolase of MT

Inhibitor	f_a	T, K	$K_A \times 10^4$, M^{-1}	N	$K_{SV} \times 10^4$, M^{-1}	$\Delta G°$, kJ/mol	$\Delta H°$, kJ/mol	$\Delta S°$, J mol^{-1} K^{-1}
Chrysin	1.09	298	4.44	0.97	5.56	−2.01	7.81	32.96
		310	3.93	0.93	28.00	−2.31		
Naringin	1.33	298	5.04	0.99	6.53	−1.70	10.46	40.80
		310	4.28	0.86	10.6	−2.10		
Quercetin	1.90	298	3.88	0.86	8.06	−2.35	-14.57	-41.03
		310	3.09	0.83	3.31	−2.91		
Gallic acid	2.97	298	4.09	0.82	1.62	−2.22	14.13	54.83
		310	4.13	0.73	2.91	−2.76		

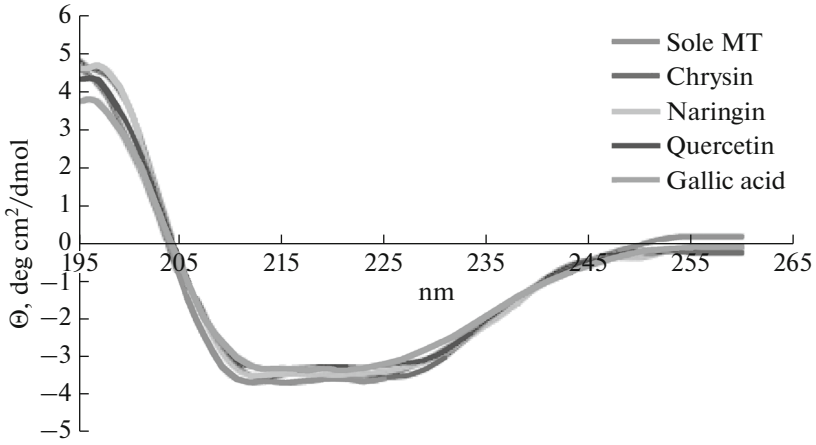

Fig. 5. CD spectra of native MT, and MT with flavonoids recorded at 298 K.

where K stands for the association constant, K_A, and R, for the gas constant. The estimated values for each parameter are represented in table.

Circular dichroism study in the far-UV region (195–260 nm), revealed that the addition of flavonoids didn't lead to significant change in the secondary structure of MT as illustrated in Fig. 5.

DISCUSSION

A variety of flavonoids have been recently isolated from different natural sources and some of them were identified as tyrosinase inhibitors. It has been suggested that flavonoid derivatives are able to limit reversibly the binding of L-DOPA to the tyrosinase active site [11]. The molecular mechanisms by which the inhibitors inhibit the enzyme activity are still in question. In the present work, therefore, we aimed to gain better understanding of the mechanisms by which several structurally similar flavonoids interact with tyrosinase using spectroscopic methods. In agreement with the previous studies [11, 22], our kinetic data clearly demonstrated that the flavonoid derivatives inhibit reversibly the diphenolase activity of MT with inhibitory potencies ranking as follows: chrysin < quercetin < naringin < gallic acid (table). These data, however, showed that these structurally similar substances are involved in different mechanisms to inhibit the enzyme activity. Chrysin, naringin and quercetin inhibited diphenolase activity competitively, while gallic acid imposed its inhibitory effect in a noncompetitive way. The competitive inhibition of chrysin, naringin and quercetin can be explicated by the structural similarity observed between these compounds and L-DOPA (Fig. 1). Additionally, comparison of the molecular structures of chrysin, naringin and quercetin with that of L-DOPA reveals that naringin is structurally bulkier than both chrysin and quercetin (Fig. 1) and has additional steric hindrance for binding of substrate to the enzyme active site. In previous study [2], some compounds, including kojic acid, exert its inhibitory effect on enzyme activity by chelating copper via 3-hydroxy-4-keto moiety. The presence of 3-hydroxy-4-keto group in the structure of quercetin may therefore suggest that this flavonoid derivative acts as copper chelator, causing competitive inhibitory effect on the diphenolase activity of MT. This hypothesis is supported by findings in previous study [2], showing that the quercetin blocked the copper-catalyzed oxidation of L-DOPA by forming a chelate with copper. Non-competitive inhibition of diphenolase activity of MT by gallic acid suggests that this inhibitor may interact with both the free tyrosinase and the tyrosinase-L-DOPA compound. The structural analysis showed that incubation of tyrosinase with flavonoid derivatives does not lead to significant change in the structure of the enzyme, as illustrated in the pattern of lines obtained from CD study (Fig. 5). On the other hand, presence of inhibitors quenches the intrinsic fluorescence of tyrosinase (Fig. 3). According to the Stern-Volmer plots (Fig. 4), which were based on data analysis of (Fig. 3), the plots of the equation (1) exhibited deviation from linear state; these results denote occurrence of both collision and static quenching processes in flavonoid interactions with the MT [17].

The negative value of ΔG° obtained from the Stern-Volmer data analysis in Table identifies that binding of flavonoids to the MT is a spontaneous process. The ΔH° and ΔS° values for chrysin, naringin, and gallic acid indicate that the force acting between these compounds and MT is mainly a hydrophobic interaction [23]. Thus, the non-polar hydrophobic groups of MT may be responsible for the main effect determining the binding of flavonoids and enzyme. The negative ΔH° and ΔS° values for quercetin emphasize the electrostatic nature of the interaction between quercetin and MT. As we observed in the kinetic data, competitive inhibition of the enzyme by quercetin might occur through chelating of copper in the enzyme active site.

The f_a and K_{SV} values can be calculated from the slope of the equations 1 and 2 (table). The values of K_{SV} at the two temperatures (298 and 310 K) showed dissimilarity and the highest values were obtained in 310 K. In addition, K_{SV} for the diphenolase activity of MT treated with flavonoids were different too, signifying that generally quenching is largely contributed by dynamic quenching, although a little static quenching part contributes to the positive divergence of the Stern-Volmer plot [24]. The magnitude of f_a for gallic acid showed to be higher than the other flavonoids (table). Parameter f_a is the part of the early fluorescence and its quantity denotes the portion of fluorescence available to quenching, which is not necessarily equivalent to the part of Trp residues available to quenching [25]. As indicated in Table, the records for n, approximate to 1 for all of the flavonoids, implying that there would have been particular binding sites for flavonoids in MT [26].

Taken together, our data clearly demonstrated that the natural flavonoids were able to inhibit reversibly the diphenolase activity of MT and could have potential applications as skin-whitening agents in cosmetic formulations.

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